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provide a better understanding and demonstrates that lncRNA-RP5 may be a potential therapeutic target in glioma.

Long non-Coding RNA RP5-821D11.7 promotes proliferation, migration, and epithelial-mesenchymal transition in glioma and glioma stem-like cells

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Abstract

Long noncoding RNA (lncRNA) has been recently revealed as a main regulatory molecule, implicating many cellular functions. Studies showed that lncRNA is abnormally expressed and involved in the progression and tumorigenesis of glioma. The present study identified a novel lncRNA associated with glioma, glioma stem-like cells (GSCs) and then revealed their potential functions. During the screening of lncRNAs, we found lncRNA RP5-821D11.7 (lncRNA-RP5) overexpress in GSCs compared to glioma cells. Lentivirus-mediated shRNA for lncRNA-RP5 was constructed and transfected into glioma cells. Transfected stable glioma cells were transplanted into nude mice and tumor growth was determined. Knockdown of lncRNA-RP5 significantly inhibits proliferation, migration and reduces epithelial-mesenchymal transition (EMT) by activating the Wnt/ β -catenin pathway. Additionally, the results showed that lncRNA RP5 knockdown enhances cell apoptosis through endoplasmic reticulum stress. Therefore, this study may



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Introduction

Malignant glioma is the most frequent and aggressive neoplasm of the brain [1]. According to World Health Organization (WHO) grading criteria, gliomas can be divided into grades I-IV[2]. LncRNAs are RNA molecules with less than 200 nucleotides that function as RNA with little or no protein encoding [3-5]. LncRNA has been implicated in playing a functional role in tumorigenesis and cancer growth [6-8]. There is increasing evidence that lncRNA participates in a diversity of many biological processes. The mechanistic role and functional diversity of lncRNAs are currently a field of research investigation [9, 10].

The Endoplasmic reticulum(ER) is a vital multifunctional eukaryotic organelle involved in lipid biosynthesis, calcium storage, protein transportation, and folding[11]. Protein misfolding in ER disturbs the ER-associated protein degradation pathway (ERAD). In ER, misfolded proteins are transported for proteasomes degradation through an ER-associated protein degradation system (ERAD). ER homeostasis disturbance may cause ER stress by ERAD[12]. Endoplasmic reticulum stress stimulates unfolded protein response (UPR), which triggers UPR-related downstream important components such as PERK, ATF, and IRE1 involved in the maintenance and re-establishment of ER stress. Furthermore, survival pathways and apoptosis have been activated after ER stress [13, 14]. However, ER stress

the UPR leads to apoptosis through the activation of caspase [15], C/EBP homologous protein (CHOP), and c-Jun NH2-terminal kinase(JNK) [16].

EMT is an essential process through which epithelial cells transform into mesenchymal cells, enhancing the metastatic phenotype of cells [17]. This increased ability of migration caused by EMT restricts the entire recurrence of tumors [18, 19]. However, the effector molecules and the signaling pathway mechanism that regulate EMT of glioma remain unclear [17]. Currently, cancer stem cell research has discovered that GSCs represent the sub-population of glioma with tumorigenic activity, EMT, and chemo-radiotherapy resistance properties [20-22]. Precisely due to these properties, glioma stem-like cells are supposed as responsible for glioma reoccurrence and therapy failure in glioma patients [23, 24]. Thus, GSCs are recognized as a target for therapeutic glioma resection and contribute to therapy resistance.

In this study, we have discovered and compared the functional mechanism of lncRNA-RP5, which enhanced EMT by activating the Wnt/ β -catenin pathway in glioma and GSCs. LncRNA-RP5 knockdown significantly inhibits proliferation, migration, and oncosphere formation, and *in vivo* tumor growth by inducing apoptosis.

Materials and Methods

Cell culture

Glioma cell lines were obtained from American Type Culture Collection (ATTC). DMEM cell culture medium (Gibco), including FBS (Gibco), penicillin, and streptomycin (100U/mL), was used for cell culture at 37°C with 5% CO₂ in a humidified atmosphere. The U87Sc and

U251Sc were isolated from U87 and U251 glioma cells by the method of serum clone formation. Medium without serum was composed of DMEM/F12 (Hyclone), 2% B27 supplement (Invitrogen), 20ug/L epidermal growth factor (EGF) (Sigma), 20ug/L basic alkaline fibroblast growth factor (bFGF) (Sigma)[25]. After 10 days, oncospheres were observed, dissociated, and passaged in a fresh medium.

Transfection

Lentivirus-mediated shRNA to knockdown LncRNA-RP5 and negative control lentiviral vectors were designed and synthesized by GenPharma (Suzhou, China). Lentivirus was packaged into HEK-293T cells and collected as per the manufacturer's instructions. U87 and U251 glioma cells were used for lentiviral particle infection, and stable cells were established using puromycin as a selection marker.

Real-Time quantitative PCR

Trizol reagent was used to extract RNA from glioma and GSCs cell lines, and stored at -80°C. RNA was transcribed into cDNA by using a reverse transcription kit (Vazyme, China). qPCR experiment was carried out using the SYBER Prime Script RT-PCR kit (Takara) on ABI-7900 real-time PCR system. Expression of the gene was calculated using 2^{-ΔΔCT} method. Primer sequences were used as follows:

LncRNA-RP5-46C24.7

Forward: 5'

GTCTGAACATCACGCCGAAC 3',

Reverse:5'

AGAACCCCTGGTATCAGTGCTAT 3'

GAPDH

Forward: 5' GCACCGTCAAGGCTGAGAAC 3',

Reverse: 5' TGGTGAAGACGCCAGTGA 3'

Cell proliferation

Lentivirus-mediated sh-RP5 Stable cells (U87, U251, U87Sc, and U251Sc) and

negative control cells were subjected to cell Counting Kit-8 (CCK8) (Biosharp, China) for proliferation study[26]. In short, 3000 cells of each group were plated in 96 well plates, and cell viability was measured at 1-4 days. The OD (Optical Density) was detected at 450nm by a microplate reader (Perkin Elmer, USA).

Migration assay

Glioma cell migration was evaluated by using a transwell chamber (Miltenyi) according to a previously reported method [27-29]. Briefly, an equal number of cells were suspended in medium (without serum), placed onto the upper chamber, and 0.6 mL of medium with FBS was added to the lower chamber. After the incubation of 24hrs, the glioma cells on the upper surface of the chamber were removed, and methanol was used for 15min to fix the cell of the lower chamber. Then cells were stained with 1% crystal violet for a half hour and counted.

Oncosphere-formation assays

Ultra-low attachment 6-well plates were used to seed 5×10³ GSCs cells/mL in a modified DMEM medium, as defined in the cell culture section, without serum supplementation[5, 8, 30, 31]. The Medium of cells was changed every 72-96 hrs. GSCs were dissociated into single cells and grew in 24 well plates at a density of 200 cells per well. After 14 days of growth, the numbers and diameters were calculated.

Flow cytometry

Cell apoptosis was determined by the Annexin V-FITC apoptosis detection kit (Vazyme Nanjing, China) following the manufacturer's protocol[12, 22, 30, 32]. Sh-RP5 cells and negative control cells were washed with PBS and then resuspended in a staining buffer. The

Annexin V-FITC and Propidium Iodide (PI) fluorescence levels were measured by flow cytometry (FCM).

Immunostaining

Four percent paraformaldehyde (PFA) was used to fix U87 and U251 sh-RP5 and NC cells at 4°C for 12hrs. Fixed cells were permeabilized in PBS with 0.3% Triton X-100 at room temperature for 20min. 10% normal goat serum (NGS) in PBS with 0.3% Triton X-100 was used to block permeabilized cells for 2hrs at room temperature. The blocked cells were placed in primary antibodies diluted in PBS with 10% NGS and 0.3% triton X-100 for 12 hrs at 4°C. Then, cells were washed and incubated with secondary antibodies for 2hrs at room temperature. A Mounting solution (Prolong® Gold antifade reagent with DAPI) was used to mount the cells. Then, the slides were examined by using Fluoview (FV3000, Olympus) confocal microscope.

Western blotting

RIPA lysis buffer containing Protease inhibitor was used for protein extraction. The BCA kit was used to measure the protein concentration of samples. As per the previously reported method, western blotting experiments were analyzed [27, 28, 33]. Protein expression was detected by using the following antibodies: EpCAM (Abcam), CD44, SOX2, Nanog, N-cadherin E-cadherin, β -catenin, phospho- β -catenin Ser33/37/Thr41, Non-phospho (Active) β -catenin Ser33/37/Thr41 (Active- β -catenin) (Cell Signaling), c-Myc, CHOP (Proteintech), and Caspase-4 ([Santa Cruz](#)). Antibodies were diluted according to specification. The Chemiluminescence method was used for the examination of protein expression.

Tumor mice model

The xenograft tumor mice model was generated by subcutaneously injected sh-RP5 (U87) and NC glioma cells. The sh-

RP5 stable and NC (5×10^6) in 200 μ L media without serum were implanted into the lateral thoracic region of BALB/c athymic nude mice (female, aged three-four weeks and 18-20g weight) supplied by the KeyGENBioTECH corp., Ltd. Tumor volume was observed by measuring length and width every week. The tumor volume was calculated using the formula: volume = (length $\times$ width²)/2.

Statistical analysis

GraphPad Prism 5.0 (GraphPad Software Inc.) was used for statistical analysis. Data were expressed as the mean $\pm$ standard deviation (SD). *P*-values less than 0.05 were considered statistically significant.

Results

LncRNA-RP5 expression up-regulated in GSCs and facilitated the proliferation

In this work, we have screened more than 200 lncRNAs and compared the expression between glioma and GSCs (data not shown). Interestingly, during the screening, we discovered that LncRNA-RP5 expression significantly up-regulates in GSCs compared to glioma (**Fig. 1A**). LncRNA-RP5 is a novel lncRNA, and the function of LncRNA-RP5 in the tumor was unknown. To confirm the non-coding potential of LncRNA-RP5, we performed the online detection with Coding Potential Assessment Tool (CPAT v2.0.0)[34]. The value for lnc00492 obtained from the CPAT database indicates that LncRNA-RP5 is a noncoding RNA (Supplementary Table 1). Furthermore, to identify the role of lncRNA-RP5 in glioma and GSCs, we silenced LncRNA-RP5 using lentivirus-

mediated short hairpin RNA (shRNAs). Notably, Knockdown assay through shRNA significantly suppressed the expression of lncRNA-RP5 in (U87 and U251) and GSCs (U87SCs and U251SCs) cell lines compared to NC (**Fig. 1B, C**). Additionally, the effect of lncRNA-RP5 knockdown on the proliferation of glioma and GSCs was detected by CCK-8 assay, which indicated that the proliferation rate was significantly inhibited in the sh-RP5 group compared to the negative control (**Fig. 1D-G**). These results indicated that lncRNA-RP5 might be involved in tumor initiation *in vitro*.

LncRNA-RP5 is required for oncosphere formation, self-renewal, and stemness of GSCs

To determine the role of lncRNA-RP5 in glioma and GSCs self-renewal, we analyzed the oncosphere formation assay. The oncosphere formation experiment

showed that negative control cells formed bigger oncospheres, while the sh-RP5 (U87SCs) cells formed a smaller oncosphere after 14 days. A similar observation was obtained for sh-RP5 (U251SCs) cells (**Fig. S1A, B**). Results showed that lncRNA-RP5 might be involved in cloning forming and self-renewal of glioma *in vitro*. lncRNA-RP5 depletion in U87SCs and U251SCs significantly reduced the expression of cancer stem cells (CSC) marker CD44 and EpCAM in sh-RP5 cells compared to the negative control. Moreover, stem cell genes Sox2 and Nanog pluripotent transcription factors favoring the maintenance of stem cells[24, 26, 35] were similarly analyzed. The Sox2 and Nanog protein expression were also reduced in lentivirus-mediated shRNA (U87SCs and u251SCs) cells compared to negative control cells. These outcomes suggested that lncRNA-RP5 may be vital in maintaining GSCs (**Fig. S2A-D**).

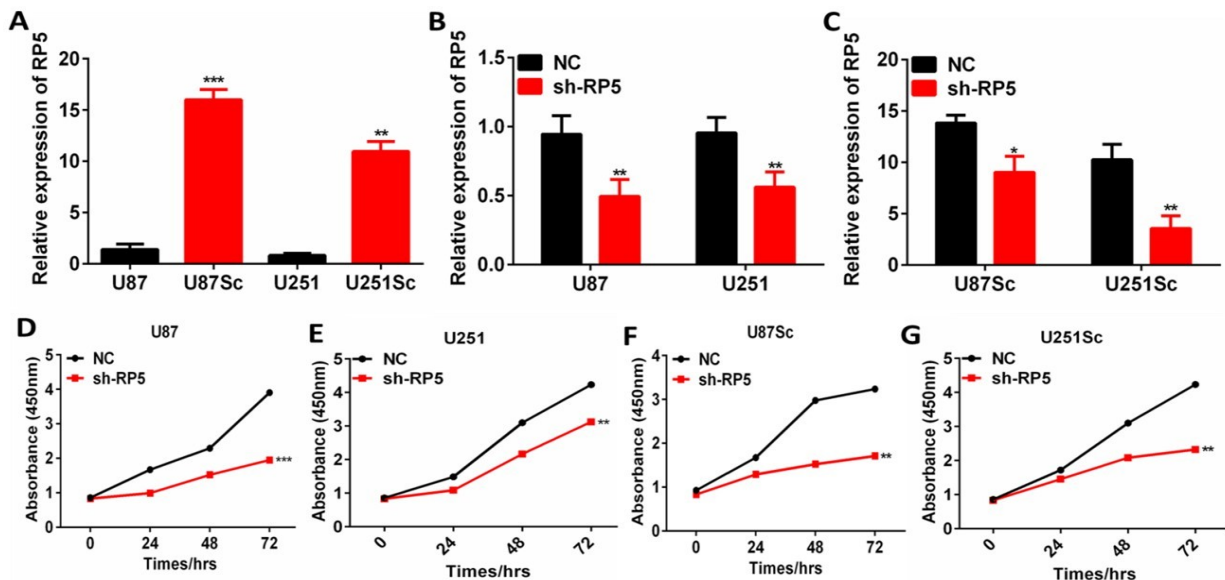


Fig. 1: LncRNARP5 up-regulated GSCs, which promotes proliferation in glioma and GSCs. Relative -RNA expression of lncRNA RP5 was validated by RT-PCR in glioma and GSCs(A), analysis showing elevated lncRNA RP5 levels in GSCs as compared to U87 and U251 cells. lncRNA RP5 is efficiently knocked down in U87 and U251 cells using a lentivirus silencing strategy. The height of the columns in the chart represents the mean expression values (B, C). lncRNA RP5 silencing reduces the proliferation of glioma and GSCs cells. Cell viability of negative control (NC) and shRNA-RP5 cells were examined using the CCK-8 assay (D-G). (**p<0.01, ***p<0.001).

LncRNA-RP5 knockdown induces apoptosis

To determine whether the depletion of lncRNA-RP5 affects apoptosis, we examined cell apoptosis through flow

cytometry (FCM). The proportion of apoptotic cells was determined by staining of cells using PI and annexin V-FITC. Cell apoptosis was significantly induced in sh-RP5 (U87 and U251) compared to negative control cells (**Fig. 2A-D**). The mechanism of enhanced apoptosis was assessed by examining the protein expression of the endoplasmic reticulum through western blotting. As shown in **Fig. 3**, compared with the

negative control group, glioma and GSCs knockdown cells displayed increased expression levels of ER apoptotic protein CHOP and cleaved caspase-4 (**Fig. 3A-E**). Furthermore, Immunostaining results also demonstrated the increased expression levels of CHOP in sh-RP5 treated cells compared to negative control cells (**Fig. 3F, G**). This data implied that the knockdown of lncRNA-RP5 could promote apoptosis in glioma cells.

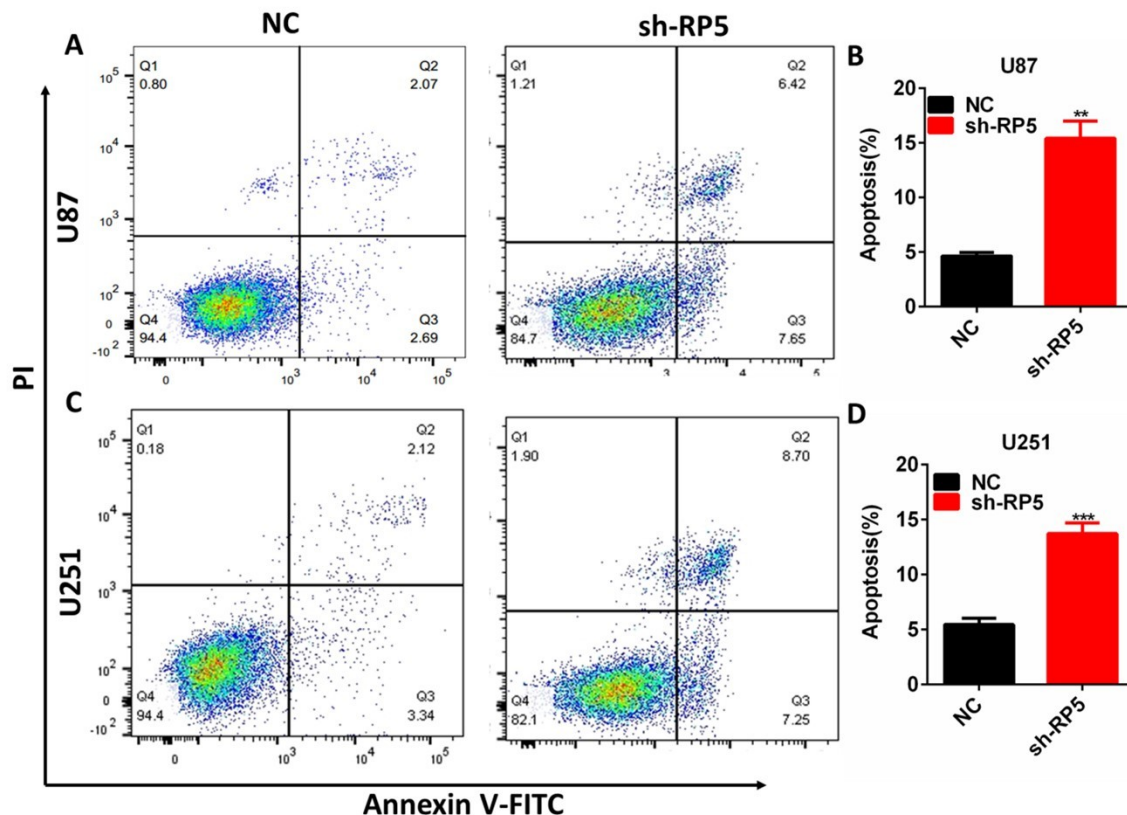


Fig. 2: LncRNA-RP5 depletion induces apoptosis in glioma cells. Cell apoptosis of U87 and U251 cells was determined by flow cytometry in sh-RP5 lentivirus stable cells and negative control (A, C). Quantitative results of apoptosis are shown in (B, D). (** $p < 0.01$ and *** $p < 0.001$).

Knockdown cells showed a lower ability of migration and EMT in glioma and GSCs

Moreover, we performed transwell assay in sh-RP5 (U87 and U251) glioma cells and the negative control group and found that sh-RP5 glioma cells have migratory ability than the negative control (**Fig. S3**). Additionally, previous experiments have shown that lncRNA-RP5 knockdown

reduces stemness properties and properties related to the EMT. To determine whether LncRNA-RP5 is involved in the EMT of GSCs, we determined the expression levels of E-cadherin, N-cadherin, and Vimentin in the negative control, and lentivirus-mediated stable sh-RP5 glioma and GSCs by western blotting. The results showed lower expression levels of E-cadherin, N-cadherin, and Vimentin after the

knockdown of lncRNA-RP5 in U87 and U251 cells, suggesting that lncRNA-RP5 might be related to EMT in glioma (**Fig. 4A-D**). Furthermore, the EMT marker's expression level, such as E-cadherin increased while N-cadherin and Vimentin

were lower in the sh-RP5 stable GSCs than the negative control GSCs (**Fig. 4E-H**). These results specify that lncRNA-RP5 knockdown can reduce the EMT of glioma and glioma-like stem cells.

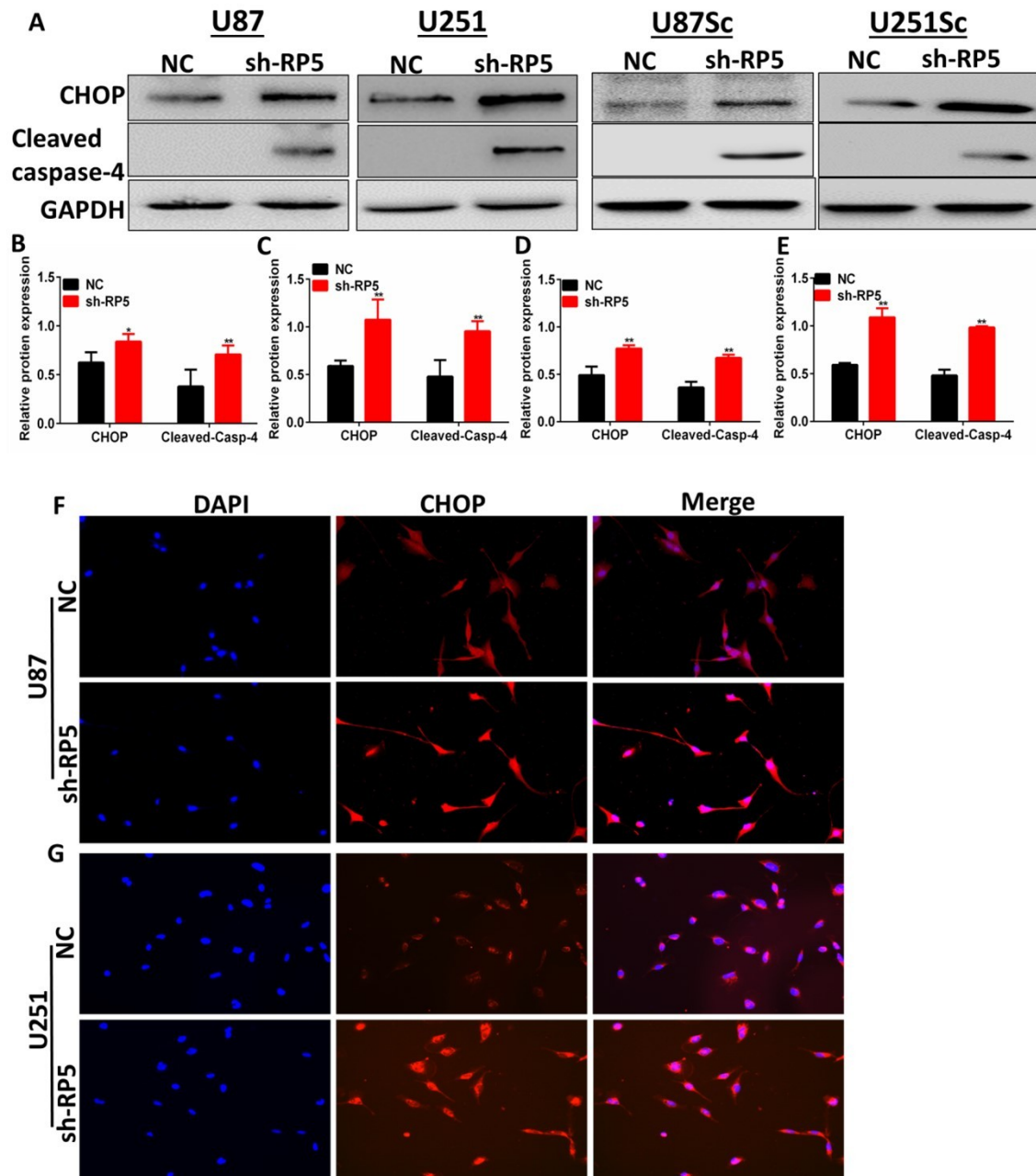


Fig. 3: Protein expressions of ER stress apoptosis markers. The expression levels of CHOP and cleaved caspase-4 were examined in glioma and GSCs cells after the knockdown of lncRNA-RP5 through lentivirus (A). Error bars represent the mean $\pm$ SD (B-E). (* $p < 0.05$ and ** $p < 0.01$). The CHOP expression level was also analyzed by immunostaining. Confocal images of U87 and U251 showed the enhanced expression of CHOP in lncRNA-RP5 knockdown cells compared to the negative control (F, G). Confocal images were captured with Fluoview, (FV3000) confocal microscope at 20X.

LncRNA-RP5 knockdown inhibits EMT through Wnt/ β -catenin signaling in GSCs

We examined the relationship between lncRNA-RP5 and the phosphorylation of β -catenin. Firstly, we analyzed the β -catenin pathway protein in glioma cell lines, and no significant change was observed in the negative control group

and sh-RP5 cells (**Fig. 5A-D**). Next, we checked in U87SCs and U251SCs. The expression level of phosphor- β -cateninSer33/37/Thr41 (p- β -catenin) was increased, and non-phospho (active) β -catenin Ser33/37/Thr41 expression was reduced in the stable sh-RP5 GSCs compared to the negative control group. In contrast,

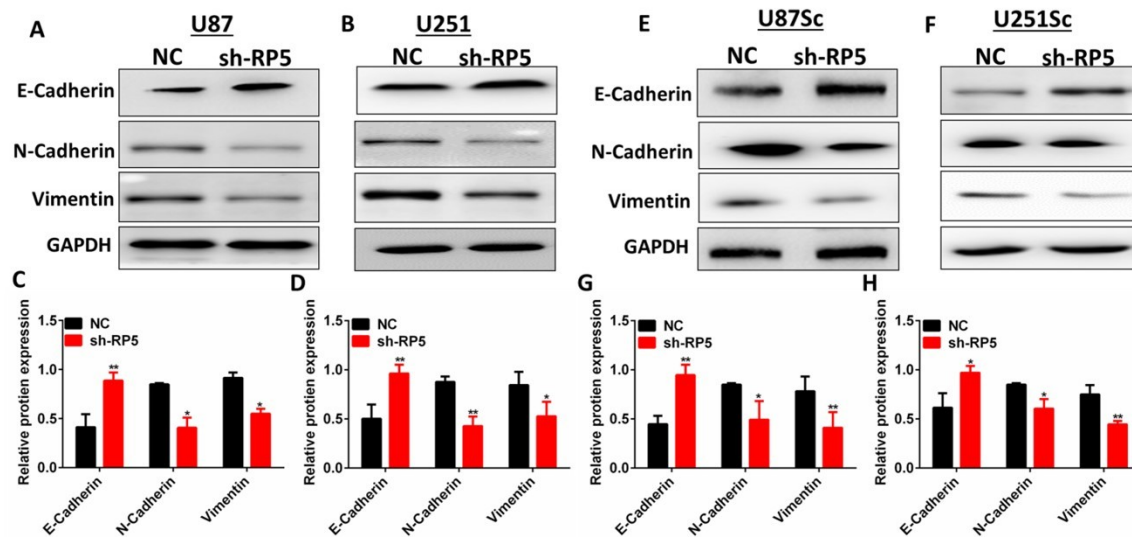


Fig. 4: EMT markers in glioma and GSC cell lines. The expression levels of E-cadherin, N-cadherin and Vimentin were examined in U87, U251, U87SCs, and U251SCs after knockdown of lncRNA-RP5 through lentivirus as compared to NC (A, B, E, F). Error bars represent the mean $\pm$ SD of three independent experiments (C, D, G, H). (*p<0.05, **p<0.01).

the total β -catenin expression level remained unchanged (**Fig. 5E-H**). Based on the previous results, we assumed that lncRNA-RP5 knockdown could reduce EMT by activating the Wnt/ β -catenin pathway. To assess this assumption, sh-RP5 (U87 and U251) glioma-like stem cells treated with the Wnt-C59 (Wnt/ β -catenin inhibitor) and found that the Wnt/ β -catenin pathway and EMT marker proteins were inhibited in sh-RP5 GSCs in a dose depended manner (**Fig. 6A-D**). Overall, we demonstrated that lncRNA-RP5 knockdown could reduce EMT and decrease migration ability by activating the Wnt/ β -catenin pathway in GSCs.

Knockdown of lncRNA-RP5 suppressed tumor growth in vivo

To examine the functional role of lncRNA-RP5 in the carcinogenesis of glioma[29, 36, 37], we investigated the role of lncRNA-RP5 on tumor growth *in vivo*. We performed a tumor xenograft experiment and found that lncRNA-RP5 knockdown significantly inhibits the growth of tumors *in vivo*. An equal number of stable knockdown of lncRNA-RP5 stable and negative control glioma stable cells was subcutaneously injected in BALB/c nude mice and after the growth of tumors, mice were sacrificed, and solid tumors were isolated and weighed. As presented in **Fig. 7** the size and weights of tumors were clearly suppressed in sh-RP5 group in comparison to the negative control group (**Fig. 7A-D**). These outcomes suggest that lncRNA-RP5 depletion reduced tumor-initiating and growth capacity.

Discussion

Glioma is one of the most lethal neoplasms, and currently, the conventional therapeutic options for glioma fail to affect glioma patients significantly [38]. Most glioma patients have shown poor prognosis and recurrence, which are thought to be related to the GSCs[24, 39, 40]. It is required to find the GSCs and discover the GSCs related treatment targets to recover glioma patients. Our findings indicated that lncRNA-RP5 could serve as a useful advanced marker of GSCs with strong stemness characteristics. CCK-8 analysis, and oncosphere formation displayed stronger malignant potency of lncRNA-RP5 in glioma and GSCs. Several

cancer gliomas stem cell markers have been reported, such as CD44 and EpCAM [41, 42]. Western blotting analysis showed that lncRNA-RP5 depletion in GSCs presented a significantly reduced expression level of these markers, demonstrating that lncRNA-RP5 may be involved in stem cell characteristics of GSCs. Furthermore, we revealed that lncRNA-RP5 also regulated the known CSCs genes such as Sox2 and Nanog. Previous studies showed that chemotherapy could disrupt the homeostasis of ER, which may induce tumor progression, therapeutic resistance, and metastasis[5, 37, 43]. Protein expression analysis through western blotting and immunostaining showed that CHOP and cleaved caspase-4 increased in glioma

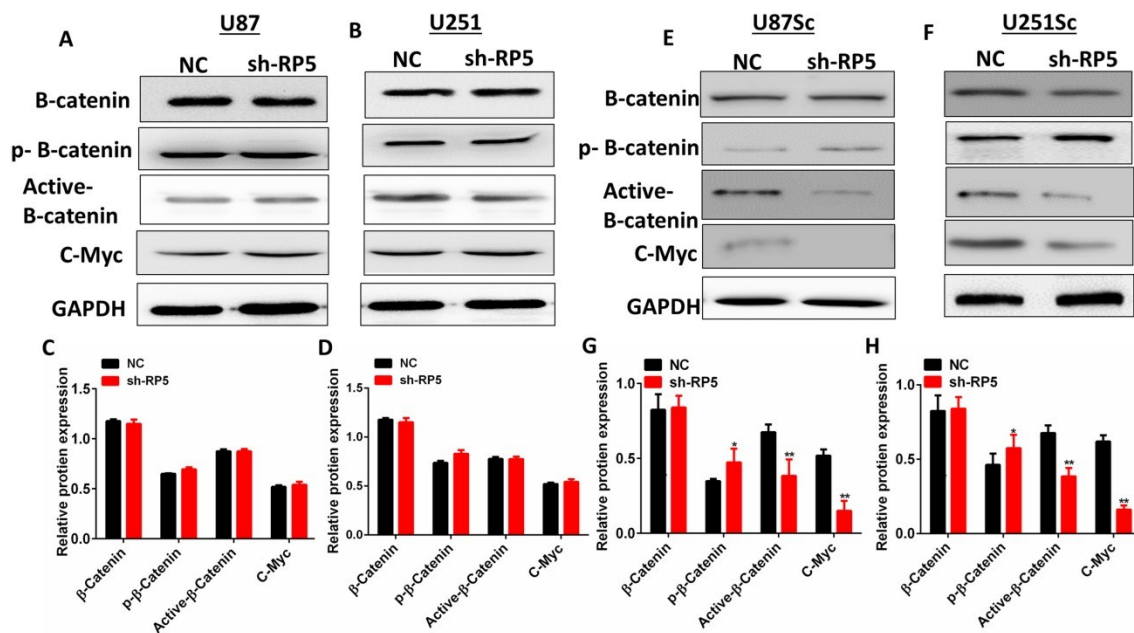


Fig. 5: β-catenin signaling protein expression in glioma and GSCs. The expression level of phospho-β-catenin Ser33/37/Thr41 (p-β-catenin), Non-phospho (Active) β-catenin Ser33/37/Thr41 (Active-β-catenin) and c-Myc was analyzed by western blotting in NC and lentivirus-mediated shRNA-RP5 cells of glioma (A-D) and GSCs (E-H). Error bars represent the mean $\pm$ SD of experiments (E-H). (* $p < 0.05$, ** $p < 0.01$).

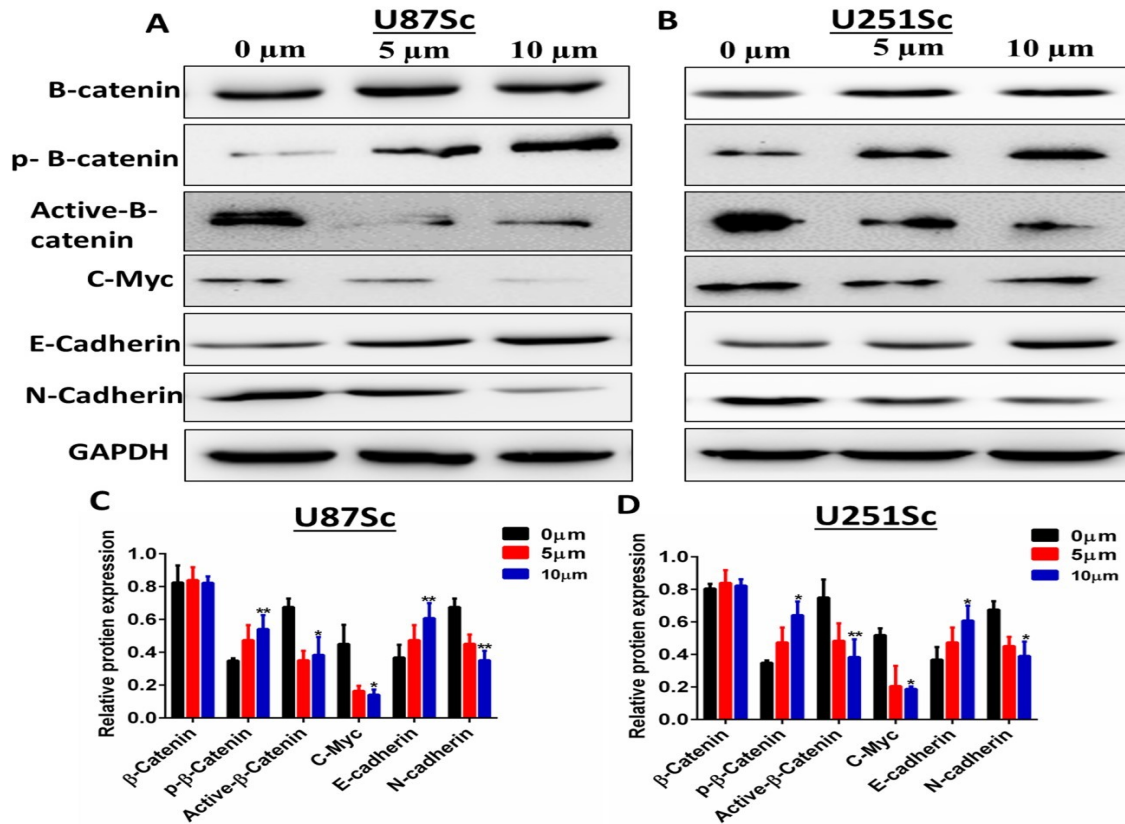


Fig. 6: EMT by activating Wnt/ β -catenin signaling in GSCs. The expression level of phospho- β -catenin Ser33/37/Thr41 (p- β -catenin), Non-phospho (Active) β -catenin Ser33/37/Thr41 (Active- β -catenin), c-Myc, E-Cadherin, N-Cadherin was analyzed by western blotting in U87, and U251 GSCs treated with the Wnt-C59 (Wnt/ β -catenin inhibitor) (A, B). Error bars represent the mean $\pm$ SD of experiments (C, D). (* $p < 0.05$, ** $p < 0.01$).

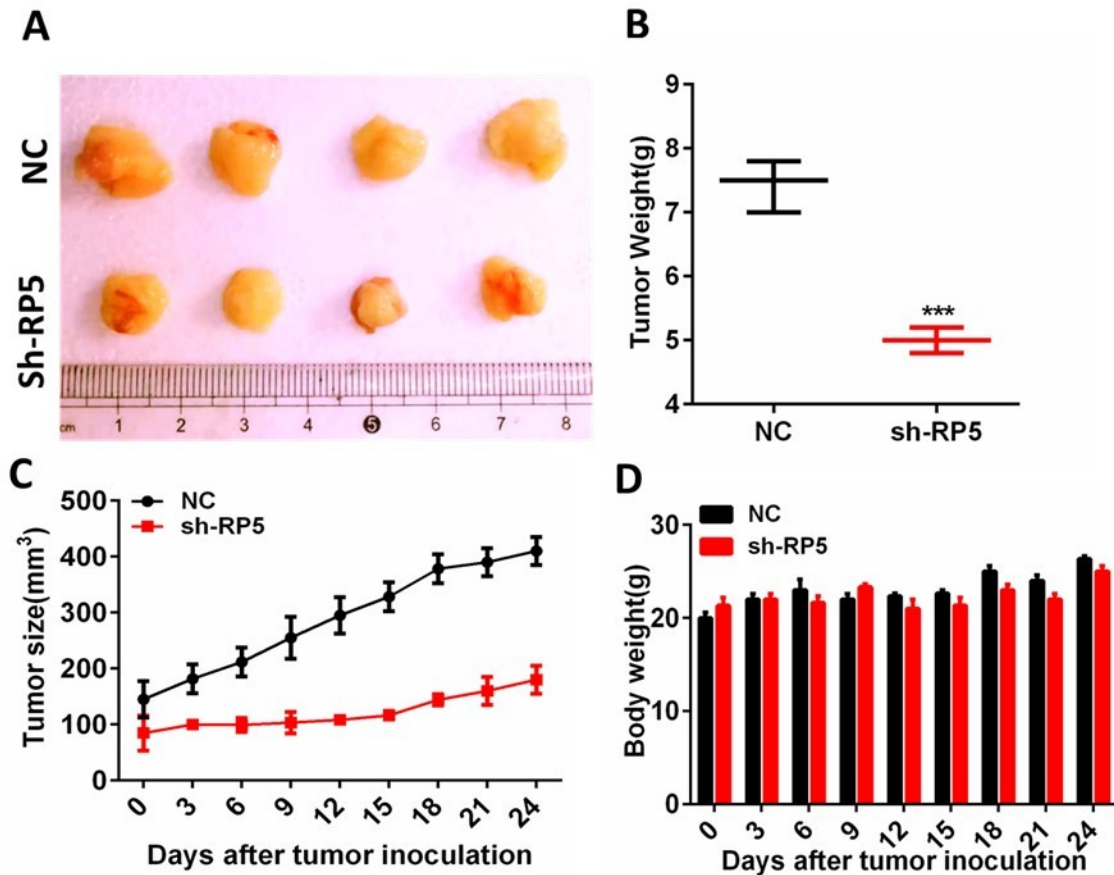


Fig. 7: *In vivo* tumor mice model. Glioma cells and negative control (NC) of shRNA-RP5(U87) were subcutaneously injected in female BALB/c nude mice. Tumors from xenografted dissected mice after 24 days were shown in the image (A). A decrease in tumor growth was noted in sh-RP5 compared to NC. Graph displaying the mean weight of resected tumors (B). Tumor volume curve showing the growth of the tumors (C), Graph presenting bodyweight of mice (D). (***) $p < 0.001$.

and GSCs after the knockdown of lncRNA-RP5. This data suggested that suppressing lncRNA-RP5 expression may induce apoptosis through ER stress pathway.

In recent years, increasing evidence suggested that the role of GSCs in glioma recurrence is a possible attribute to its phenotype of EMT [21, 44, 45]. Thus, it is important to explore the effector molecules and signaling pathways to regulate the EMT in GSCs. Furthermore, recent studies showed that ER stress is also a responsible factor inducing EMT[46-48]. These results suggested that lncRNA-RP5 may regulate apoptosis via the ER stress pathway in glioma. In this work, for the very first time, we have revealed that lncRNA-RP5 is related to

and significantly affects migration in glioma and GSCs. Additionally, the EMT markers (E-cadherin, N-cadherin, and vimentin) were regulated by lncRNA-RP5, demonstrating that lncRNA-RP5 is an important effector molecule that drives EMT of glioma as well as GSCs. Further, we explored the mechanism of EMT in GSCs; our findings demonstrated that lncRNA-RP5 could activate the Wnt/ β -catenin pathway by inhibiting the β -catenin phosphorylation. Moreover, the effect of the Wnt-C59 inhibitor was also analyzed, which inhibited Wnt/ β -catenin pathway in GSCs. Based on the earlier results, we can conclude that lncRNA-RP5 can encourage EMT through the Wnt/ β -catenin pathway. *In vivo* experiments also showed that the

knockdown of lncRNA-RP5 significantly inhibited the growth of the tumor as well as prolonged the survival time of xenograft mice. Our research presented that lncRNA-RP5 is involved in cell proliferation, migration, stem cell properties, and tumorigenicity of glioma. In conclusion, this study unveils that lncRNA-RP5 knockdown induces ER mediated apoptosis in glioma and GSCs. Moreover, lncRNA-RP5 is a functional GSCs marker, drives EMT by activating the Wnt/ β -catenin pathway in GSCs, and may be involved in recurrence and poor prognosis of glioma. Therefore, we believe that further research related to lncRNA-RP5 will explore novel therapeutic approaches to glioma.

Author contributions

M.Y and S.S designed and performed the experiments. M.Y and S.S analyzed the data. M.Y and K.A.S wrote the manuscript. M.Y and S.S contributed ideas and discussed results. K.A.S supervised the whole project. All authors read and approved the manuscript.

Ethics Approval and Consent

All animal experiments were performed under the guidelines of the National Institute of Health, 2006, China and the Animal Care Research Advisory Committee of Southeast University, Nanjing, Jiangsu, China.

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Conflict of interest

The authors declare no conflict of interest.

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